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PRIORITY DOCUMENT		
	Application #	10/657,703
	Conf. #	6086
	Filing Date	9 September 2003
	First Inventor	PEBAY et al.
	Art Unit	1653
	Examiner	
	Docket #	P08048US00/BAS

LETTER

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

S I R:

Applicant hereby claims the priority dates of the attached documents under the provisions of 35 U.S.C. 119.

Respectfully submitted,

Date: June 28, 2004

B. Aaron Schulman
Registration No. 31,877

STITES & HARBISON PLLC
Transpotomac Plaza
1199 North Fairfax Street, Suite 900
Alexandria, Virginia 22314
(703) 739-4900

Patent Office
Canberra

I, JONNE YABSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. 2003901313 for a patent by ES CELL INTERNATIONAL PTE LTD as filed on 21 March 2003.

WITNESS my hand this
Ninth day of September 2003

J R Yabsley

JONNE YABSLEY
TEAM LEADER EXAMINATION
SUPPORT AND SALES

P/00/009
Regulation 3.2

AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

APPLICANT: **ES CELL INTERNATIONAL PTE LTD**

INVENTION TITLE: **UNDIFFERENTIATED CELLS**

The invention is described in the following statement:

INHIBITING SPONTANEOUS DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS

Technical Field

The present invention relates to methods for inhibiting spontaneous differentiation of stem cells. It also relates to media useful in propagating stem cells in an undifferentiated state.

Background Art

In general, stem cells are undifferentiated cells which can give rise to a succession of mature functional cells. For example, a haematopoietic stem cell may give rise to any of the different types of terminally differentiated blood cells. Embryonic stem (ES) cells are derived from the embryo and are pluripotent, thus possessing the capability of developing into any organ, cell type or tissue type or, at least potentially, into a complete embryo. ES cells may be derived from the inner cell mass of the blastocyst, which have the ability to differentiate into tissues representative of the three embryonic germ layers (mesoderm, ectoderm, endoderm), and into the extra-embryonic tissues that support development.

Human embryonic stem cells (hES cells) are pluripotent cell lines derived from the inner cell mass of the blastocyst. These cells have the ability to differentiate into functional tissues representative of the three embryonic germ layers (mesoderm, ectoderm, endoderm), and into extra-embryonic tissues that support development. Because of their ability to generate these different cellular fates, hES cells are considered to be of great potential for future therapies.

However, during routine culture *in vitro*, established hES cell lines have a tendency to spontaneously differentiate. Because the pluripotency of these cells is associated with their undifferentiated state, it is desirable to find a way to limit this spontaneous differentiation. Contrary to what is seen in mouse embryonic stem cells, leukemia inhibitory factor (LIF) does not prevent the spontaneous differentiation of hES cells [1]. Thus, a common way to grow and then to maintain hES cells in an optimum state is to cultivate

them on feeder layers, which are constituted by primary mouse embryonic fibroblasts (MEF), in media supplemented with high doses of fetal calf serum.

However, serum contains a wide variety of biologically active compounds that might have the potential to adversely affect hES cell growth and differentiation. Furthermore, there is a biosafety issue if cells cultured in animal serum are subsequently used for implantation in a human or for the production of a biological therapeutic.

With regard to these issues and in order to establish a serum-free culture system to grow hES cells, it is of great importance to identify the specific factors in serum that are responsible for its beneficial effect on the growth of hES cells. Thus, alternative approaches to traditional culture systems are desirable, such as the use of a serum replacement medium such as Knockout Serum Replacement [2, 3].

Sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA) are two small bioactive lysophospholipids, present in serum (at concentration of up to 1 and 5 μM respectively) [4], released by activated platelets, which act on a wide range of cell types derived from the three developmental germ layers. Most of the effects of these lysophospholipids seem to be mediated by specific lysophospholipid G-protein coupled receptors (LPL receptors) previously named endothelial differentiation gene (Edg) receptors.

Up to now, eight distinct mammalian LPL/Edg receptors have been identified: $S1P_1$/Edg-1, $S1P_2$/Edg-5, $S1P_3$/Edg-3, $S1P_4$/Edg-6 and $S1P_5$/Edg-8 are specific for S1P while LPA_1/Edg-2, LPA_2/Edg-4 and LPA_3/Edg-7 are specific for LPA (for reviews see [5, 6]). Each of these receptors is coupled to at least one G protein and can activate or inhibit specific signalling pathways. For instance, all these receptors are coupled to $G_{i/o}$ proteins (for review see [5, 6]).

By activating notably these $G_{i/o}$ proteins, S1P and LPA can stimulate the extracellular-signal-regulated kinases 1 and 2 (ERK1/2), which are members of the mitogen-activated protein (MAP) kinase family, and thus are involved in regulation of major cellular events, such as cell proliferation or differentiation. S1P and LPA are potent biological agents involved in

numerous cell events, such as proliferation, differentiation, death or migration (for review see [5]) since the very early stages of development.

S1P stimulates mammalian angiogenesis, at least via $S1P_1$ and $S1P_2$ [7-10]. Thus, $S1P_1$ knockout mice show impaired blood vessel maturation. Moreover, in the zebrafish, S1P is required for normal heart development [11]. Thus, in these animals, the mutation of the gene *mil* that encodes the S1P receptor Mil (very similar to the mammalian $S1P_2$ receptor) impairs migration of cardiac progenitor cells [11].

On the other hand, LPA seems to be mainly involved in neurogenesis [12]. For instance, LPA, probably via LPA_1, stimulates cell cycle-morphological changes and cell migration of cultured cortical neuroblasts. Moreover, LPA, probably via LPA_2, regulates the migration of post-mitotic neurons to their final destination. Last but not least, LPA_1 knockout mice present abnormal cerebral cortices and olfactory bulbs, probably due to impaired development, demonstrating LPA_1 is essential for a normal brain development [13].

Within serum, Platelet-Derived Growth Factor (PDGF) is a major protein growth factor that has been widely described as a potent mitogen of numerous kinds of cells. PDGF has also been shown to induce chemotaxis, actin re-organization, and to prevent apoptosis. This growth factor belongs to a family of dimeric isoforms of polypeptide chains, A, B, C and D that act through different tyrosine kinase receptors: PDGFR-α and PDGFR-β.

S1P and PDGF have additional effects that induce biological responses. Thus S1P and PDGF are able to regulate smooth muscle cell migration, proliferation and vascular maturation. Moreover, Hobson *et al.* (2001), and Rosenfeld *et al.* (2001) demonstrated that PDGF-stimulated cell motility is $S1P_1$-dependent in HEK 293 cells and MEF [14, 15] while Kluk *et al.* (2003) showed that this effect was independent of $S1P_1$ in vascular smooth muscles and MEF [16]. Last but not least, it is now proposed that PDGF is able to stimulate the enzyme sphingosine kinase, which leads to an increase in S1P intracellular concentration [17], an effect that would be responsible for PDGF-induced proliferation in Swiss 3T3 cells [17] and vascular smooth muscle cells [18].

The discussion of documents, acts, materials, devices, articles and the like is included in this specification solely for the purpose of providing a context for the present invention. It is not suggested or represented that any or all of these matters formed part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia before the priority date of each claim of this application.

Description of the Invention

The present inventors investigated the role of the LPL receptor agonists S1P, dihydro S1P and LPA, and the ligand of a class III tyrosine kinase receptor, PDGF, in modulating the fate of hES cells in culture.

The present inventors have established that hES cells are target cells for S1P, dihydro S1P, LPA and PDGF, through expression of the LPL receptors, PDGFR-α and PDGFR-β and through stimulation of ERKs by these agonists. Moreover the present inventors have found that S1P and PDGF slightly inhibit the spontaneous differentiation of hES cells while co-incubation with both S1P and PDGF strongly reduces the spontaneous differentiation of hES cells. These findings provide a basis for the establishment of a serum free culture medium for stem cells and in particular hES cells.

Throughout the description and claims of this specification, the word "comprise" and variations of that word, such as "comprising" and "comprises" are not intended to exclude other additives, steps or integers.

In a first aspect the present invention provides a method for modulating spontaneous differentiation of a stem cell having a lysophospholipid (LPL) receptor and PDGF receptors, which method comprises incubating the stem cell in the presence of an agonist of the LPL receptors and a ligand of a class III tyrosine kinase receptor.

In a second aspect the present invention provides a method for modulating spontaneous differentiation of a stem cell, which method comprises incubating the stem cell in the presence of a ligand of a class III tyrosine kinase receptor.

In a third aspect the present invention provides a method for modulating spontaneous differentiation of a stem cell having a lysophospholipid (LPL) receptor, which method comprises incubating the stem cell in the presence of an agonist of the LPL receptor.

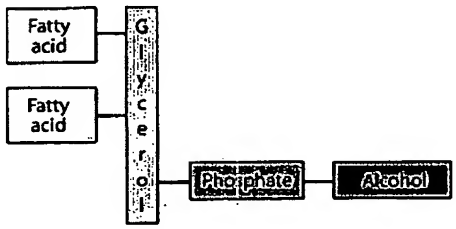
Sphingosine-1-phosphate (S1P), an agonist of the LPL receptors has the ability to at least partially inhibit the spontaneous loss of stem cell phenotype in cell culture. It has also been found that the method does not affect the ability of stem cells to proliferate.

Preferably, the LPL receptor is selected from the group including S1P1, S1P2 and S1P3.

As used herein the term "modulating the differentiation of a stem cell" includes the inhibition or enhancement of cellular differentiation. The term also includes partial inhibition or enhancement of cellular differentiation. In a preferred form of the method, the modulation is inhibition of differentiation.

Typically the agonist is a phospholipid.

As used herein, the term "phospholipid" refers to a molecule that includes a backbone attached to two fatty acid moieties and a phosphate group. The backbone on which the fatty acid molecules are attached is variable and may be based on glycerol or sphingosine for example. A diagram of a generic phospholipid is shown below.

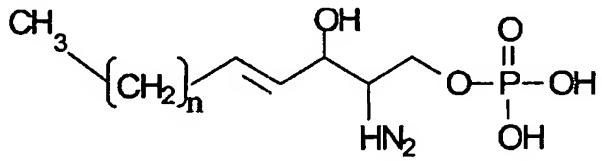


The term "lysophospholipid" refers to a phospholipid molecule where one of the fatty acid chains has been removed. The removal of a fatty acid chain may be accomplished by treatment of the phospholipid with an enzyme such as phospholipidase A2.

The phospholipid or lysophoholipid may have a sphingosine backbone, and particularly, the lysophospholipid may be a phosphorlyated

amino alcohol. Preferably the agonist is selected from the group consisting of S1P, dihydro S1P, LPA, PAF and SPC.

In a highly preferred form of the invention the lysophospholipid is sphingosine-1-phosphate (S1P) or a functional equivalent thereof. S1P is a small bioactive phospholipid, present in serum, released by activated platelets, which has the following structure:



The skilled person will understand that bioactive molecules such as phospholipids and lysophospholipids may be altered in a number of ways and still retain biological activity. Accordingly, the scope of the present invention includes altered forms of phospholipids and lysopholipids that retain their LPL receptor agonist activity. The scope of the present invention also includes synthetic peptidic agonists of the LPL receptors.

The skilled person will be familiar with methods which can be applied to testing phospholipids or lysophospholipids for the ability to modulate the ability of a stem cell to differentiate. Suitable methods are found herein, and include reactivity with antibodies such as GCTM-2 which are directed to stem cell specific markers, and simple morphological evaluation of cells by light microscopy.

For example, the effect of the agonist on the differentiation of stem cells into neuronal or endodermal lineages may be studies by analysis of marker expression as shown in PCT AU01/00278 and AU01/00735.

The phospholipid or lysophospholipid may be extracted from a biological source such as serum. In addition, mast cells and monocytes are able to produce S1P while adipocytes produce LPA, however the main source of LPA and S1P is activated platelets. Alternatively, the phospholipid may be synthesised by procedures well known in the field of organic chemistry.

Preferably, cells that have been exposed to a LPL receptor agonist are not substantially negatively affected in their ability to proliferate. Therefore,

an advantage of the methods and compositions described herein is that it is possible to expand a population of hES cells without leading to a loss in pluripotency. Methods for determining the proliferative capability of a hES cell will be known by the skilled person and include detection of the cell proliferation marker PCNA as described herein.

Typically the ligand is a PDGF.

The tyrosine kinase receptor may be PDGFR-α or PDGFR-β.

In a preferred embodiment the PDGF is PDGFab or PDGFbb which both bind to the two types of receptors.

The method may also include use of TNF alpha, NGF (nerve growth factor), muscarinic acetylcholine agonists, serum or phorbol esters - which again are compounds that have additive or synergistic effects with S1P.

The stem cell may be derived from foetal tissue or adult tissue.

The stem cell is typically an ES cell. Preferably the stem cell is a hES cell. As used herein the term "embryonic stem cell" means a cultured cell line derived from preimplantation stages of development capable of differentiation into tissues representative of all three embryonic germ layers.

Theses cells:

- express SSEA-3,SSEA-4, TRA 1-60, GCTM-2, alkaline phosphatase and Oct-4
- Grow as flat colonies with distinct cell borders
- Differentiate into derivatives of all three embryonic germ layers
- Are feeder cell dependent (feeder cell effect on growth not reconstituted by conditioned medium from feeder cells or by feeder cell extracellular matrix)
- Are highly sensitive to dissociation to single cells and show poor cloning efficiency even on a feeder cell layer
- Do not respond to Leukemia Inhibitory Factor

In a third aspect the present invention provides a serum free medium useful for modulating spontaneous differentiation of a stem cell having a LPL receptor, comprising an agonist of the LPL receptor and a ligand of a class III tyrosine kinase receptor.

In a fourth aspect the present invention provides a serum free medium useful for modulating spontaneous differentiation of a stem cell, comprising a ligand of a class III tyrosine kinase receptor.

The medium is useful in propagating stem cells such as human embryonic stem cells in an undifferentiated state.

Typically the ligand is a PDGF.

The tyrosine kinase receptor may be PDGFR-α or PDGFR-β.

In a preferred embodiment the PDGF is PDGFab or PDGFbb.

The medium may also include TNF alpha, NGF (nerve growth factor), muscarinic acetylcholine agonists, serum or phorbol esters - which again are compounds that have additive or synergistic effects with S1P.

Typically the agonist is a phospholipid.

Preferably the agonist is selected from the group consisting of S1P, LPA, PAF, dihydro S1P and SPC.

The stem cells may be derived from foetal tissue or adult tissue.

The stem cells are typically embryonic stem cells.

Preferably the stem cells are from embryonic tissue.

Typically the stem cells are of human origin.

The base medium is typically a standard serum free medium that is supplemented with phospholipids and ligand as well as a buffering agent. A suitable buffering agent is 25mM Hepes.

The medium is of use in inhibiting the differentiation of pluripotent stem cells.

The cell culture medium may be based on any of the base media known in the art useful for the growth and/or maintenance of stem cells such as hES cells. Such media include but are not limited to Dulbecco's Modified Eagles Medium (DMEM), KNOCKOUT-DMEM or HES medium. In a preferred form of the invention the medium is based on DMEM supplemented with insulin, transferrin and selenium.

The optimal concentration of LPL agonist in the medium may be determined by routine experimentation. However, in a preferred form of the invention the agonist is present in the medium at a concentration of from 0.1 μM to 10μM where the agonist is S1P. In a highly preferred form of the

invention the agonist is present in the medium at a concentration of about 10μM. It would be expected that the optimum concentration will vary according to a number of parameters including the species of agonist, the line of stem cells being cultured, the base medium used, and other culture conditions such as temperature, carbon dioxide concentration, and humidity.

The optimal concentration of ligand in the medium may be determined by routine experimentation. However, in a preferred form of the invention the ligand is present in the medium at a concentration of from 1 ng/ml to 20ng/ml where the ligand is either PDGFab or PDGFbb. In a highly preferred form of the invention the ligand is present in the medium at a concentration of 20 ng/ml. Again, it would be expected that the optimum concentration will vary according to a number of parameters including the species of agonist, the line of stem cells being cultured, the base medium used, and other culture conditions such as temperature, carbon dioxide concentration, and humidity.

The skilled person understands that it is often necessary to culture hES cells on feeder cells, and the present invention contemplates methods including the use of such feeder cells. The concentration of agonist may also need to be optimised according to the feeder cell line used.

In a fifth aspect the present invention provides a stem cell grown and/or maintained in a cell culture medium of the invention.

Cells of the present invention will find many uses in biology and medicine. The properties of pluripotentiality and immortality are unique to ES cells and enable investigators to approach many issues in human biology and medicine for the first time. ES cells potentially can address the shortage of donor tissue for use in transplantation procedures, particularly where no alternative culture system can support growth of the required committed stem cell. However, it must be noted that almost all of the wide ranging potential applications of ES cell technology in human medicine-basic embryological research, functional genomics, growth factor and drug discovery, toxicology, and cell transplantation are based on the assumption that it will be possible to increase the proliferation and therefore grow ES cells on a large scale, to introduce genetic modifications into them, and to direct their differentiation.

The present invention provides a method of producing a population of proliferating undifferentiated stem cells from a stem cell which method comprises incubating the stem cell in the presence of an agonist of the LPL receptor and a ligand of a class III tyrosine kinase receptor.

The present invention also provides a method of producing a population of proliferating undifferentiated stem cells from a stem cell which method comprises incubating the stem cell in the presence of a ligand of a class III tyrosine kinase receptor.

The present invention further provides a method of producing a population of proliferating undifferentiated stem cells from a stem cell which method comprises incubating the stem cell in the presence of an agonist of the LPL receptor.

These methods therefore provide for the expansion of stem cell populations.

The invention also provides a population of undifferentiated stem cells produced by at least one of these methods.

Preferably, the LPL receptor is selected from the group including S1P1, S1P2 and S1P3.

Typically the agonist is a phospholipid.

Preferably the agonist is selected from the group consisting of S1P, dihydro S1P, LPA, PAF and SPC.

In a highly preferred form of the invention the lysophospholipid is sphingosine-1-phosphate (S1P) or a functional equivalent thereof.

Typically the ligand is a PDGF.

The tyrosine kinase receptor may be PDGFR-α or PDGFR-β.

In a preferred embodiment the PDGF is PDGFab or PDGFbb which both bind to the two types of receptors.

The ligand may also be TNF alpha, NGF (nerve growth factor), muscarinic acetylcholine agonists, serum or phorbol esters - which again are compounds that are known to act through the class III tyrosine kinase receptors.

The stem cell may be derived from foetal tissue or adult tissue.

The stem cell is typically an ES cell. Preferably the stem cell is a hES cell.

Another aspect of the present invention is a method of treating or preventing a disorder of stem cell differentiation including administering to an animal in need thereof a composition containing an agonist of a LPL receptor and a ligand of a class III tyrosine kinase receptor.

The present invention also provides a method of treating or preventing a disorder of stem cell differentiation including administering to an animal in need thereof a composition containing a ligand of a class III tyrosine kinase receptor.

The agonist is typically a phospholipid. The phospholipid may be a lysophospholipid and may have a sphingosine backbone. Preferably the agonist is selected from the group consisting of S1P, dihydro S1P, LPA, PAF and SPC. S1P and dihydro S1P are lysophospholipids with a sphingosine backbone, as is SPC, while LPA is a lysophosphospholipid with a glycerol backbone, and PAF is a phospholipid with a glycerol backbone.

The tyrosine kinase receptor may be PDGFR-α or PDGFR-β and the ligand a PDGF.

In a preferred embodiment the PDGF is PDGFab or PDGFbb.

The method may also include use of TNF alpha, NGF (nerve growth factor), muscarinic acetylcholine agonists, serum or phorbol esters - which again are compounds that have additive or synergistic effects with S1P.

Also provided is a pharmaceutical composition comprising a class III tyrosine kinase receptor ligand and a LPL receptor agonist. The composition may also include use of TNF alpha, NGF (nerve growth factor), muscarinic acetylcholine agonists, serum or phorbol esters - which again are compounds that have additive or synergistic effects with S1P.

A skilled person will be able to provide formulations and dosage forms of the agonist. Furthermore, the optimum dosage for a given clinical situation could be determined by routine experimentation.

Abbreviations

dH-S1P: dihydro-sphingosine-1-phosphate; EDG: endothelial differentiation gene; ERK: extracellular signal-regulated kinase; MAP kinase: mitogen-activated protein kinase; MEF: mouse embryonic fibroblasts; hES cells: human embryonic stem cells; LPA: lysophosphatidic acid; LPL: lysophospholipid; PAF: platelet activated factor; PCNA: proliferating cell nuclear antigen; PDGF: platelet-derived growth factor; PDGFR: platelet-derived growth factor receptor; PTX: *Pertussis* Toxin; S1P: sphingosine-1-phosphate; SPC: sphingosylphosphorylcholine; SPK: sphingosine kinase.

Brief Description of the Accompanying Figures

Figure 1: LPL and PDGF receptor mRNAs are expressed in HES cells. RT-PCR experiments were performed using mRNA isolated from HES cells using specific primers for human LPL receptors (A, B) and PDGFR-α (alpha) and PDGFR-β (beta) (C). In each case, experiments were conducted either in the presence (+) or absence (-) of reverse transcriptase. The RT-PCR products were separated by electrophoresis on 1.5% agarose gel and revealed by ethidium bromide fluorescence. Molecular sizes (in bp) of the products were calculated using 1 kB plus DNA ladder markers (M). These data are representative of results from at least 3 independent experiments, each carried out on mRNAs prepared from different cultures of HES cells.

Figure 2: PDGFR-α and PDGFR-β are expressed in HES cells. HES cells grown in the presence of serum (A, D) were immunostained with either PDGFR-α (C) or PDGFR-β (F). The same fields were also stained with Hoechst 33342 (B, E). These data are representative of at least 3 independent experiments, each performed in triplicate.

Figure 3: S1P, LPA and PDGF stimulate ERKs phosphorylation in hES cells. Western blots experiment were performed using protein lysate from hES cells. Cells were pre-treated or not with U0126 (30 μM, 1 hr) and incubated for 5 min in the absence (C, control) or presence of S1P (10 μM), LPA (50 M) or PDGF (20 ng/ml). The phosphorylation of Erk1 and Erk2 (P-Erk1 and P-Erk2) was assessed by immunoblotting with a polyclonal anti-active MAP kinase as described in Materials and Methods. After a stripping

procedure, the same blots were reprobed with a monoclonal anti-MAP kinase, allowed the detection of Erk1 and Erk2. ThESe data are representative of results from at least 3 independent experiments.

Figure 4: S1P and PDGF inhibit the spontaneous differentiation of HES cells. (A) HES cells grown with MEF, before the depletion of serum from the medium. (B, C, D, E) HES cells grown without serum after 8 days, in the absence (B) or in the presence of S1P (10 μM) (C), PDGF (20 ng/ml) (D), S1P (10 μM) plus PDGF (20 ng/ml) (E). (F) HES cells grown without serum, in the presence or in the absence (control) of S1P (10 μM), PDGF (20 ng/ml), S1P (10 μM) plus PDGF (20 ng/ml). In A-E, data are representative of at least 3 independent experiments. In F, data expressed as percentages of alkaline phosphatase activity in absence of serum (% of control), are the means ± SEM of at least 3 independent experiments, each run in triplicate.

Figure 5: S1P and PDGF inhibit the spontaneous differentiation of hES cells independently of MEF. HES cells mechanically dissociated and cultivated for 4 days in the absence (C, control) or presence of S1P (10 μM) or/and PDGF (20 ng/ml) in a media depleted in serum. (A) Quantification of the number of GCTM2+ cells. (B) Quantification of the number of PCNA+/GCTM2+ cells. These data are the mean ± SEM of results obtained in at least 3 independent experiments.

Figure 6: dihydro-S1P and PDGF-BB mimic the effect of S1P and PDGF. (A) hES cells grown without serum, in the presence or in the absence (control) of S1P (10 μM), dihydro-S1P (DHS1P, 10 μM), PDGF (20 ng/ml), S1P (10 μM) plus PDGF (20 ng/ml), dihydro-S1P (10 μM) plus PDGF (20 ng/ml). (B) hES cells grown without serum, in the presence or in the absence (control) of S1P (10 μM), PDGF-AA (20 ng/ml), PDGF-BB (20 ng/ml), S1P (10 μM) plus PDGF-AA (20 ng/ml), S1P (10 μM) plus PDGF-BB (20 ng/ml). The data expressed as percentages of alkaline phosphatase activity in absence of serum (% of control), are the means ± SEM of at least 3 independent experiments, each run in triplicate.

Figure 7 shows cells expressing mRNA for SPK1 and SPK2 showing the probable expression of the enzymes as well as the stem cell markers Oct-4, and Crypto.

The invention will now be more fully described with reference to the following non-limiting Examples.

Best Method and Other Methods of Carrying out the Present Invention

Cell culture.

hES-3 cells were cultured as previously described [1], in presence or in absence of mitotically inactivated MEF feeder layer. Culture media consisted of DMEM (without sodium pyruvate, glucose 4500 mg/l) supplemented with β-mercaptoethanol 0.1 mM, NEAA 1 %, glutamine 2 mM, Hepes 25 mM, penicillin 50 U/ml, streptomycin 50 mg/ml and 20 % fetal bovine serum (Hyclone, Logan, UT, USA). In some experiments, the day following the plating, the cells were incubated with the different agents in medium without serum. Media was changed the 2nd day following plating and then every 2 days. S1P, sphingosine and C6-ceramide were obtained from Biomol (Plymouth Meeting, PA, USA) and were dissolved in methanol. LPA was obtained from Sigma (Castle Hill, NSW, Australia) and was dissolved in ethanol. Extemporaneal dilutions of all lipids were made in water containing 0.1 % fatty acid-free bovine serum albumin (BSA) (Sigma). Human PDGF-AB, PDGF-AA, PDGF-BB were from PreproTech Inc. (Rocky Hill, NJ, USA).

RT-PCR experiments.

Cells were washed with PBS and hES colonies were removed by treatment with protease (10mg/ml). Purified mRNA was extracted from HES cultures using Dynabeads® Oligo $(dT)_{25}$ (Dynal, Oslo, Norway), according to the supplier's instruction. RT was performed using superscript II Reverse

Transcriptase (Life technologies), according to the supplier's protocol. The cDNA samples were amplified by PCR with sens and antisens primers (synthesis performed by Sigma Genosys, Castle Hill, NSW, Australia) previously designed by others for the specific detection of mouse (data not shown) or human LPPs and PDGF receptor DNA target sequences (Table 1). Standard PCR reactions were performed using Taq DNA polymerase (Biotech International Ltd, Perth, WA, Australia). Absence of contaminating genomic DNA was checked by control reactions with mRNA not been treated with reverse transcriptase. PCR experiments were run with the following steps: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 sec, different annealing temperature depending on the primer pairs (Table 1) for 2 min, extension at 74°C for 2 min, a final extension at 74°C for 7 min. The specific amplified DNA fragments were sized by electrophoresis on 1.5 % (w/v) agarose gel, stained with ethidium bromide and photographed. The amplicons were purified and sequenced. Experiments were performed on hES-2 and hES-3.

TABLE 1 : sense and antisense primers

Gene	sense and antisense primers	Size (bp)	Annealing temp (°C)	References
$S1P_1$	CCACAACGGGAGCAATAACT	480	52	[2]
	GTAAATGATGGGGTTGGTGC			
$S1P_2$	CCAATACCTTGCTCTCTCTGGC	502	52	[2]
	CAGAAGGAGGATGCTGAAGG			
$S1P_3$	TCAGGGAGGGCAGTATGTTC	505	52	[2]
	CTGAGCCTTGAAGAGGATGG			

$S1P_4$	CGGCTCATTGTTCTGCACTA	701	52	[2]
	GATCATCAGCACCGTCTTCA			
$S1P_5$	TTCTGATACCAGAGTCCGGG	460	52	[2]
	CAAGGCCTACGTGCTCTTCT			
LPA_1	GCTCCACACACGGATGAGCAACC	621	56	[3]
	GTGGTCATTGCTGTGAACTCCAGC			
LPA_2	AGCTGCACAGCCGCCTGCCCCGT	775	56	[3]
	TGCTGTGCCATGCCAGACCTTGTC			
LPA_3	CCATAGCAACCTGACCAAAAAGAG	482	56	[3]
	TCCTTGTAGGAGTAGATGATGGGG			
PDGFRα human	ATCAATCAGCCCAGATGGAC	891	58	[4]
	TTCACGGGCAGAAAGGTACT			
PDGFRβ	AATGTCTCCAGCACCTTCGT	698	58	[4]
Human	AGCGGATGTGGTAAGGCATA			
Crypto	CAGAACCTGCTGCCTGAATG	185	55	
	GTAGAAATGCCTGAGGAAACG			
SPK-1	ACCCATGAACCTGCTGTCTC	227	55	
	CAGGTGTCTTGGAACCCACT			
SPK-2	TGGCAGTGGTGTAAGAACC	200	55	
	CAGTCAGGGCGATCTAGGA			
Oct-4	CGTTCTCTTTGGAAAGGTGTTC	320	55	[5]
	ACACTCGGACCACGTCTTTC			

Immunofluorescence.

In some experiments, hES-3 cells plated onto 8-well chamber slides, with or without MEF, were fixed in ethanol the day after plating. In others, hES-3 cells were mechanically dissociated, in order to obtain a monolayer culture and then plated onto 8-well chamber slides without MEF and were fixed in ethanol 4 days after the first treatment. Immunostaining was performed using the following antibodies: anti human PDGFR-α or PDGFR-β (R&D Systems Inc.), GCTM-2, and/or PCNA (Chemicon, Boronia, VIC, Australia), TRA-1-60, Oct-4. Nuclei were evidenced by Hoechst-33342. Slides were mounted and observed by fluorescent microscopy with a Leica microscope at X10, X20 and X40. Specificity was verified by the absence of any staining in the negative controls. In some experiments, cells were counted to determine the ratio of GCTM-2 positive (GCTM2+), PCNA positive (PCNA+) and GCTM2+/PCNA+ cells within the global population.

GCTM-2 quantification.

hES-3 cells plated with MEF, were fixed in ethanol and immunostained with GCTM-2 and then with an alkaline phosphatase-coupled secondary antibody (Dako). The activity of alkaline phosphatase was detected by adding a solution of 4-nitrophenyl phosphate (Roche, Mannheim, Germany), followed by reading the optical density (OD) at 405 nm. In order to validate the technique as a relevant indicator of the proportion of GCTM-2 positive cells, standard curves were done with the teratocarcinoma cell line GCT27C4, known to express GCTM-2. This showed a linear correlation between the number of cells and the OD read at 405 nm (data not shown).

Western blot analysis.

HES-3 cells plated without MEF for 24 hrs were depleted of serum for a further 18 hrs. Cells pre-treated or not with U0126 (Sigma, 30 μM, 1 hr), were incubated in the presence of the different agents for 5 min and were lysed by removal of the supernatants and addition of a reducing loading buffer containing 1 mM sodium orthovanadate (Sigma). Protein lysates

(approx. 80 μg) were separated by SDS-polyacrylamide gel electrophoresis (10 % polyacrylamide, w/v), transferred to nitrocellulose (Hybond-nitrocellulose, Amersham) and immunoblotting was carried out using rabbit polyclonal anti-active mitogen-activated protein (MAPK) antibodies raised against a dually phosphorylated MAPK peptide (Promega, Madison, WI, USA). Peroxidase-coupled secondary antibody (Dako) was detected by exposure of autoradiographic films in the presence of a chemiluminescent detection reagent (ECL, Amersham). Stripping of antibodies was achieved and membranes were then reprobed with rabbit polyclonal anti-ERK1/2 antibodies (Promega), and then with peroxidase-coupled secondary antibodies (Dako). Membranes probed with either rabbit polyclonal anti-active p38 (Promega) or rabbit polyclonal anti-active JNK (Promega) antibodies were also performed, using the same procedure as described above.

Teratoma formation in SCID mice.

At the time of routine passage, clumps of about 200 cells with an undifferentiated morphology were harvested as previously described, and injected into the testis of four- to eight-week-old SCID mice. Six to seven weeks later, the resulting tumors are fixed in neutral buffered formalin 10%, embedded in paraffin, and examined histologically after hematoxylin and eosin staining.

Protein quantification.

hES-3 cells were lysed and the amount of proteins was determined using a colorimetric assay based on the Bradford dye-binding test (Bio-Rad Laboratories, Regents Park, NSW, Australia).

Statistical analysis.

Each set of experiments was performed at least 3 times (*n* refers to number of independent experiments performed on different cell cultures).

Data are expressed as the mean ± SEM. Significance of the differences was evaluated by using the ANOVA followed by Student-Newman Keuls test. Values of $P < 0.001$, 0.01 and 0.05 were considered significant and were respectively indicated by ***, ** and *.

RESULTS

hES cells (Figure 1A) expressed mRNA transcripts for three S1P receptors: $S1P_1$, $S1P_2$ and $S1P_3$ and for each of LPA receptors: LPA_1, LPA_2 and LPA_3 (Figure 1B), while these cells did not express mRNA for $S1P_4$ and $S1P_5$ (data not shown). hES cells also expressed mRNA transcripts for PDGFR-α (Figure 1C) and PDGFR-β (Figure 1C) as well as the corresponding proteins, as revealed by immunostaining (Figure 2). MEF expressed $S1P_1$, $S1P_2$, $S1P_3$, LPA_1 and LPA_2, PDGFR-α and PDGFR-β but neither $S1P_4$, $S1P_5$ nor LPA_3 (data not shown), as previously shown by others [6-8]. Because the MAP kinases ERKs are implicated in cell proliferation and differentiation, we examined the effects of S1P, LPA and PDGF on their activation in hES cells. After 5 min, S1P, LPA and PDGF stimulated the phosphorylation of ERKs in hES cells (Figure 3), an effect that was totally inhibited in presence of the MEK inhibitor U0126 (30 μM) (Figure 3). Interestingly, C6-ceramide (10 μM) and sphingosine (10 μM), two S1P-analogs were ineffective to stimulate ERKs (data not shown).

We next wondered if S1P, LPA and PDGF could modulate the fate of hES cells. When hES cells were grown on MEF, in a serum-free culture media, they spontaneously differentiated. As shown in Figure 4, after 8 days in such conditions (control), the colonies were bigger than those observed before the removal of serum (Figure 4A) and hES cells gave rise to different kinds of cells (Figure 4B). After 8 days, LPA (up to 50 μM) did not have an

obvious effect on growth of the colonies, as ascertained by morphological (data not shown) whilst in the presence of either S1P (10 μM) or PDGF (20 ng/ml), the colonies appeared flatter and less differentiated as compared to the control condition (Figure 4C, 4D). Thus, after 8 days of treatment, when GCTM-2 levels of cells were quantified by measuring the activity of alkaline phosphatase, cells treated with S1P or PDGF were respectively 16.6 ± 4.1 % (n=7) and 16.6 ± 7.0 % (n=7) more GCMT2+ than the control cells (Figure 4F). Strikingly, the co-incubation of both S1P (10 μM) and PDGF (20 ng/ml) induced a strong inhibition of spontaneous differentiation, not observed in the presence of one or the other agent (Figure 4E) with a higher percentage of GCTM2+ cells of 40.1 ± 7.5 % (n=7) than in the control cells (Figure 4F). As GCTM-2 is a stem cell marker, these results suggest that the combination of PDGF and S1P in a serum-free culture media strongly prevents the spontaneous differentiation of hES cells.

In order to identify the effects of S1P and PDGF on hES cells, we carried out experiments in which we forced the cells to differentiate, by 1) mechanically dissociating them before plating and 2) growing them in the absence of MEF and serum. S1P or/and PDGF were added to the culture medium and their effects on differentiation and proliferation were quantified by immunostaining the cells with PCNA, a marker of proliferation, and GCTM-2 (Figure 5). After 4 days in medium without serum, most of the control cells were differentiated, with only 30.8 ± 7.7 % (n=13) of GCTM2+ cells (Figure 5A). By contrast, when either S1P (10 μM) or PDGF (10 ng/ml) was added to the medium, 47.9 ± 3.8 % (n=13) or 53.7 ± 13.2 % (n=3) of the cells respectively were GCTM2+, and 53.7 ± 3.5 % (n=3) of the cells were GCTM2+ in presence of both S1P and PDGF. Within the hES cell population, a large proportion expressed PCNA, showing that the majority of these stem

cells still proliferated (Figure 5B). However, there was no statistically significant difference in the proliferating rate of hES cells between the control cells and the ones treated with either S1P or/and PDGF (Figure 5B). Altogether, these data suggest that S1P and PDGF mostly act on the differentiation of hES cells grown in the absence of serum rather then on the proliferating state of hES cells. Moreover, because the hES cells were cultivated in absence of MEF, these experiments clearly show that S1P and PDGF are able to directly target the hES cells.

We next investigated the effect of dihydrosphingosine-1-phosphate (dihydro-S1P, 10 μM), an S1P analogue that can only mimics the receptor-dependent effects of S1P. By measuring the GCTM2 levels of the cells, we showed that the effect seen in presence of S1P and PDGF was mimicked by dihydro-S1P and PDGF (125.7 ± 9.7 % of control (n=3)), demonstrating that S1P's effect is receptor-dependent (Figure 6A). We then investigated which isoform of PDGF was the most potent in inhibiting the spontaneous differentiation of HES cells. When added with S1P, the isoform BB was the most potent (182.0 ± 26.0 % of control (n=2)), followed by AB (125.7 ± 9.7 % of control (n=3)), while AA elicited little effect (120.5 ± 4.5 % of control (n=2)) (Figure 6B).

Passaging

The hES cells have successfully been passaged through at least 10 passages in PDGF and S1P, with no serum.

After passage3 the cells have stained positive for the stem cell markers GCTM-2, Oct-4 and TG30.

After passage 6 the cells were transfered into the testis capsules of SCID mice for teratoma formation. Observation of teratomas confirms that these cells are in fact still stem cells.

After passage 7 the cells expressed mRNA for SPK1 and SPK2 showing the probable expression of the enzymes as well as the stem cell markers Oct-4, and Crypto (see Figure 7).

After passage 8: karyotyping of HES cells – is being carried out to show that these cells when cultured in serum free conditions with PDGF and S1P have maintained a normal karyotype.

DISCUSSION

Since hES cells spontaneously differentiate in culture, a phenomenon that leads to a loss of their pluripotency, the identification of the compounds that are able to prevent this differentiation is of particular interest. In this study, we describe for the first time that hES cells are targets of S1P, LPA and PDGF.

As revealed by RT-PCR analysis, these cells express the mRNA for the receptors $S1P_1$, $S1P_2$, $S1P_3$, LPA_1, LPA_2 and LPA_3.. Referring to studies performed in rodent or in human, these receptors are widely expressed in the body (for reviews see [9,10]). The absence of expression of $S1P_4$ and $S1P_5$ in these cells is in accordance with the fact that these receptors seem to be mostly expressed in highly differentiated tissues, such as lymphoid tissue for $S1P_4$ [11] and in brain's white matter for $S1P_5$ [12]. Moreover, hES cells express the PDGF-receptors α and β, as revealed by RT-PCR and immunostaining. In hES cells, the addition of both PDGF and S1P inhibit very strongly the

spontaneous differentiation, suggesting that these two molecules do cross talk. These combined effects could be attributed to the fact that 1) PDGF stimulates the formation of intracellular S1P which would then act as a second messenger, for instance in the regulation of calcium homeostasis [13] and in the suppression of apoptosis, as shown in fibroblasts [14] and other cell types [15,16], but up to now the intracellular targets of S1P remain unclear; 2) S1P acts extracellularly through its receptors, and thus activates different intracellular signalling pathways, such as the MAP kinases, involved in cell proliferation. The presence of both intracellular and extracellular S1P might then lead to a stronger inhibition of differentiation than the ones observed in presence of either S1P or PDGF. Also reported is a new cross link between PDGF and S1P signals, in which both molecules need to be present. Such a mechanism has recently been described for the first time by Katsuma *et al.* (2002) [17]in mesangial cells.

As shown by others, S1P, LPA and PDGF receptors are expressed in MEF [7] and these molecules are able to regulate multiple signalling pathways. Thus, Ishii *et al.* (2001) demonstrated that in these cells, S1P activates phospholipase C, inhibits the production of cAMP and activates Rho [7]. In MEF, PDGF stimulates migration. The effect observed in presence of PDGF and S1P on HES cells might be in part due to an effect through the MEF.

S1P, LPA and PDGF are all present in serum from different species, including bovine and human. However, the concentration of these molecules varies from one species to another. Thus, it is believed that this could explain the commonly observed phenomenon with current cell culturing techniques where there is not only species dependant variation in the performance of serum used to supplement cell culture systems but also intra-species batch to batch variations as well.

Altogether, these data suggest that within the lipids and the proteins present into the serum, both S1P and PDGF are key elements in the regulation of spontaneous differentiation of hES cells. Identification of compounds having an ability to inhibit differentiation allows the design of simple culture media more suitable for hES cell propagation. Moreover, in a therapeutic view, it is important to determine compounds that allow cultivation of hES cells in a serum-free environment.

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CLAIMS

1. A method for modulating spontaneous differentiation of a stem cell having a lysophospholipid (LPL) receptor, which method comprises incubating the stem cell in the presence of an agonist of the LPL receptor and a ligand of a class III tyrosine kinase receptor.

2. A method for modulating spontaneous differentiation of a stem cell, which method comprises incubating the stem cell in the presence of a ligand of a class III tyrosine kinase receptor.

3. A method for modulating spontaneous differentiation of a stem cell having a lysophospholipid (LPL) receptor, which method comprises incubating the stem cell in the presence of an agonist of the LPL receptor.

4. A method according to any one of claims 1 and 3 wherein the LPL receptor is selected from the group including S1P1, S1P2, S1P3.

5. A method according to any one of claims 1 to 4 wherein the modulation is inhibition of differentiation.

6. A method according to any one of claims 1 to 5 wherein the agonist is a phospholipid.

7. A method according to claim 6 wherein the agonist is selected from the group consisting of S1P, dihydro S1P, LPA, PAF and SPC.

8. A method according to any one of claims 1 to 7 wherein the tyrosine kinase receptor is PDGFR-α or PDGFR-β.

9. A method according to claim 8 wherein the ligand is a PDGF.

10. A method according to claim 9 wherein the PDGF is PDGFab or PDGFbb.

11. A method according to any one of claims 1 to 10 which also includes use of TNF alpha, NGF (nerve growth factor), muscarinic acetylcholine agonists, serum or phorbol esters.

12. A method according to any one of claims 1 to 11 wherein the stem cell is derived from foetal tissue or adult tissue.

13. A method according to claim 12 wherein the stem cell is an ES cell.

14. A method according to claim 13 wherein the stem cell is a hES cell.

15. A serum free medium useful for modulating spontaneous

differentiation of a stem cell having a LPL receptor, comprising an agonist of the LPL receptor and a ligand of a class III tyrosine kinase receptor.

16. A serum free medium useful for modulating spontaneous differentiation of a stem cell, comprising a ligand of a class III tyrosine kinase receptor.

17. A medium according to claim 15 wherein the LPL receptor is selected from the group including S1P1, S1P2, S1P3.

18. A medium according to any one of claims 15 to 17 wherein the modulation is inhibition of differentiation.

19. A medium according to any one of claims 15 to 18 wherein the agonist is a phospholipid.

20. A medium according to claim 19 wherein the agonist is selected from the group consisting of S1P, dihydro S1P, LPA, PAF and SPC.

21. A medium according to any one of claims 15 to 20 wherein the tyrosine kinase receptor is PDGFR-α or PDGFR-β.

22. A medium according to claim 21 wherein the ligand is a PDGF.

23. A medium according to claim 22 wherein the PDGF is PDGFab or PDGFbb.

24. A medium according to any one of claims 15 to 23 which also includes TNF alpha, NGF (nerve growth factor), muscarinic acetylcholine agonists, serum or phorbol esters.

25. A medium according to any one of claims 15 to 24 wherein the stem cell is derived from foetal tissue or adult tissue.

26. A medium according to claim 25 wherein the stem cell is an ES cell.

27. A medium according to claim 26 wherein the stem cell is a hES cell.

28. A medium according to any one of claims 15 to 27 wherein the base medium is a standard serum free medium that is supplemented with phospholipids and ligand as well as a buffering agent.

29. A medium according to claim 28 wherein the buffering agent is 25mM Hepes.

30. A medium according to claim 28 wherein the medium is based on DMEM supplemented with insulin, transferrin and selenium.

31. A medium according to claim 28 wherein the agonist is S1P and is present in the medium at a concentration of from 0.1 μM to 10μM.

32. A medium according to claim 31 wherein the agonist is present in the medium at a concentration of about 10μM.

33. A medium according to claim 28 wherein the ligand is present in the medium at a concentration of from 1 ng/ml to 20ng/ml where the ligand is either PDGFab or PDGFbb.

34. A medium according to claim 33 wherein the ligand is present in the medium at a concentration of 20 ng/ml.

35. Use of the medium of any one of claims 15 to 34 in propagating stem cells such as human embryonic stem cells in an undifferentiated state.

36. A stem cell grown and/or maintained in a cell culture medium according to any one of claims 15 to 34.

37. A method of treating or preventing a disorder of stem cell differentiation including administering to an animal in need thereof a composition containing an agonist of a LPL receptor and a ligand of a class III tyrosine kinase receptor.

38. A method of treating or preventing a disorder of stem cell differentiation including administering to an animal in need thereof a composition containing a ligand of a class III tyrosine kinase receptor.

39. A method according to claim 38 wherein the LPL receptor is selected from the group including S1P1, S1P2, S1P3.

40. A method according to any one of claims 37 to 39 wherein the modulation is inhibition of differentiation.

41. A method according to any one of claims 37 to 40 wherein the agonist is a phospholipid.

42. A method according to claim 41 wherein the agonist is selected from the group consisting of S1P, dihydro S1P, LPA, PAF and SPC.

43. A method according to any one of claims 37 to 42 wherein the tyrosine kinase receptor is PDGFR-α or PDGFR-β.

44. A method according to claim 43 wherein the ligand is a PDGF.

45. A method according to claim 44 wherein the PDGF is PDGFab or PDGFbb.

46. A method according to any one of claims 37 to 45 which also includes use of TNF alpha, NGF (nerve growth factor), muscarinic acetylcholine agonists, serum or phorbol esters.

47. A method according to any one of claims 37 to 46 wherein the stem cell is derived from foetal tissue or adult tissue.

48. A method according to claim 47 wherein the stem cell is an ES cell.

49. A method according to claim 48 wherein the stem cell is a hES cell.

50. A pharmaceutical composition comprising a class III tyrosine kinase receptor ligand and a LPL receptor agonist.

51. A pharmaceutical composition comprising a class III tyrosine kinase receptor ligand and a LPL receptor agonist which also includes use of TNF alpha, NGF (nerve growth factor), muscarinic acetylcholine agonists, serum or phorbol esters.

52. A method of producing a population of proliferating undifferentiated stem cells from a stem cell which method comprises incubating the stem cell in the presence of an agonist of the LPL receptor and a ligand of a class III tyrosine kinase receptor.

53. A method of producing a population of proliferating undifferentiated stem cells from a stem cell which method comprises incubating the stem cell in the presence of a ligand of a class III tyrosine kinase receptor.

54. A method of producing a population of proliferating undifferentiated stem cells from a stem cell which method comprises incubating the stem cell in the presence of an agonist of the LPL receptor.

55. A method according to any one of claims 52 to 54 wherein the LPL receptor is selected from the group including S1P1, S1P2 and S1P3.

56. A method according to any one of claims 52 to 55 wherein the agonist is a phospholipid.

57. A method according to any one of claims 52 to 56 wherein the agonist is selected from the group consisting of S1P, dihydro S1P, LPA, PAF and SPC.

58. A method according to any one of claims 52 to 57 wherein the agonist is sphingosine-1-phosphate (S1P) or a functional equivalent thereof.

59. A method according to any one of claims 52 to 58 wherein the ligand is a PDGF.

60. A method according to any one of claims 52 to 59 wherein the tyrosine kinase receptor is PDGFR-α or PDGFR-β.

61. A method according to any one of claims 52 to 60 wherein the PDGF is PDGFab or PDGFbb.

62. A method according to any one of claims 52 to 61 which includes the use of TNF alpha, NGF (nerve growth factor), muscarinic acetylcholine agonists, serum or phorbol esters.

63. A method according to any one of claims 52 to 62 wherein the stem cell is derived from foetal tissue or adult tissue.

64. A method according to claim 63 wherein the stem cell is an ES cell.

65. A method according to claim 64 wherein the stem cell is a hES cell.

66. A population of undifferentiated stem cells produced by at least one of the methods according to claims 52 to 65.

DATED THIS 21ST DAY OF MARCH 2003

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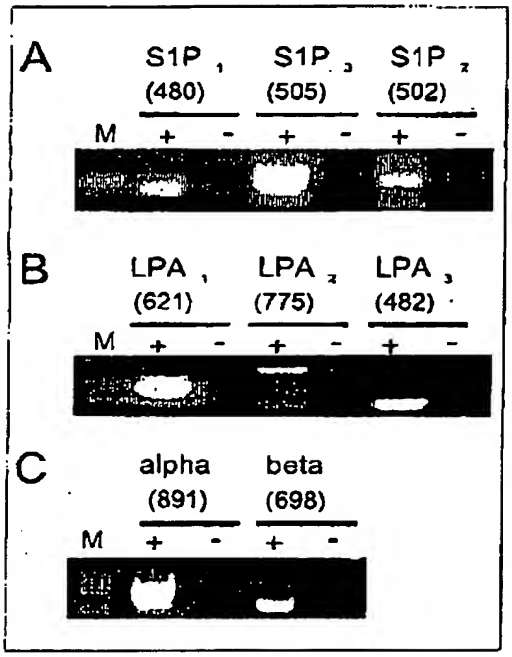
A
S1P 1
(480)
S1P 3
(505)
S1P 2
(502)
M + - + - + -
B
LPA 1
(621)
LPA 2
(775)
LPA 3
(482)
M + - + - + -
C
alpha
(891)
beta
(698)
M + - + -

FIG 1

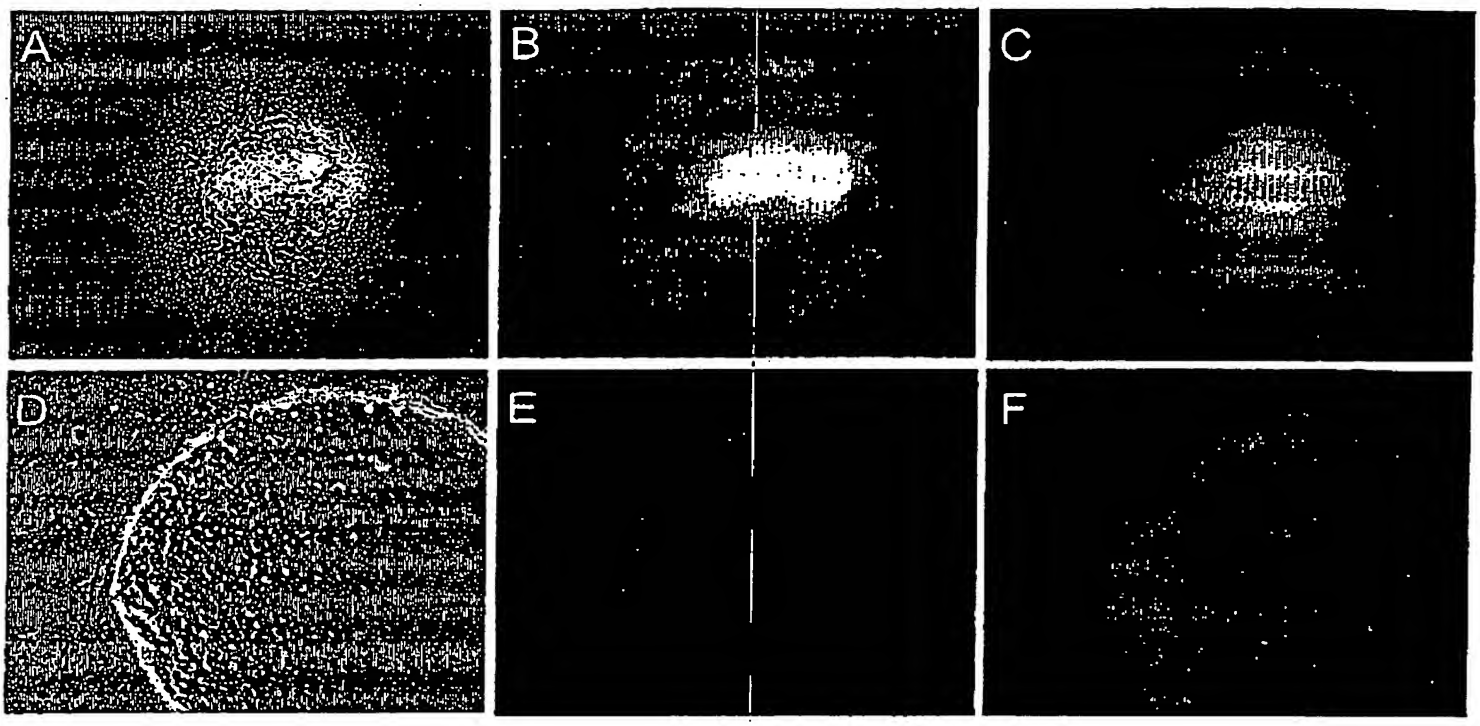

FIG 2.

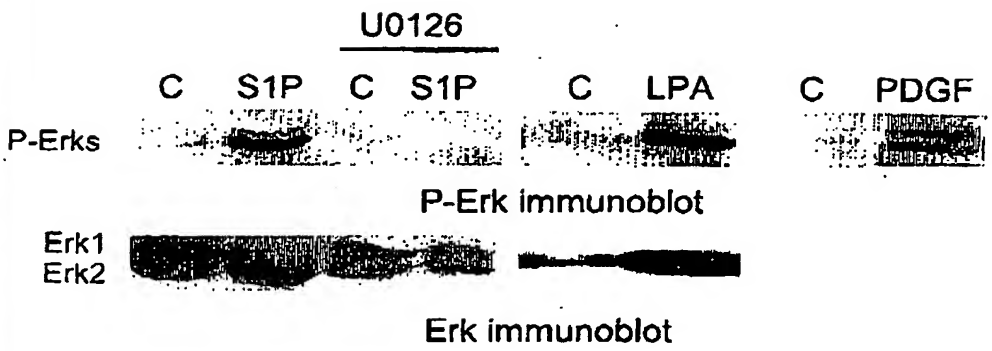
U0126
C
S1P
C
S1P
C
LPA
C
PDGF
P-Erks
P-Erk immunoblot
Erk1
Erk2
Erk immunoblot

FIG 3

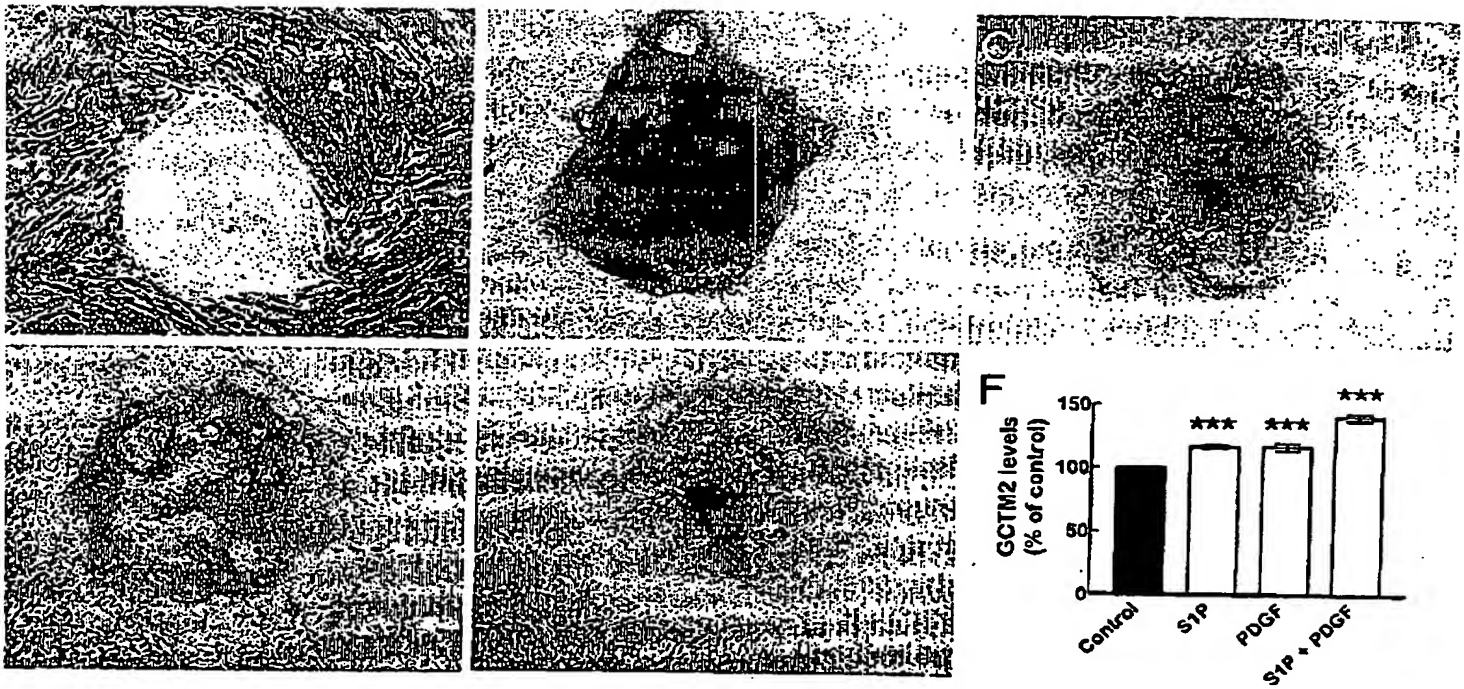
F
GCTM2 levels
(% of control)
150
100
50
0
***
***
***
Control
S1P
PDGF
S1P + PDGF

FIG 4

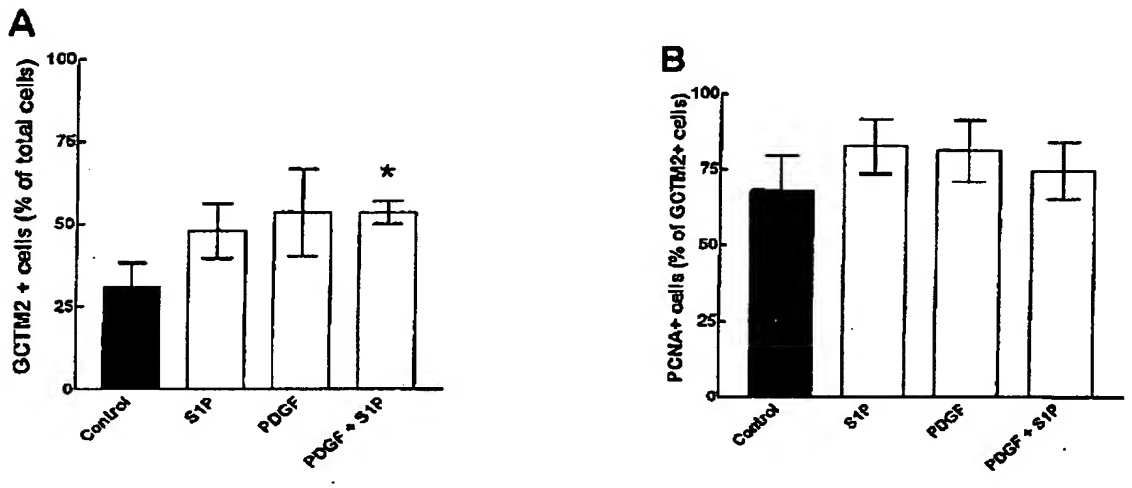
A
GCTM2 + cells (% of total cells)
100
75
50
25
0
*
Control
S1P
PDGF
PDGF + S1P
B
PCNA+ cells (% of GCTM2+ cells)
100
75
50
25
0
Control
S1P
PDGF
PDGF + S1P

FIG 5

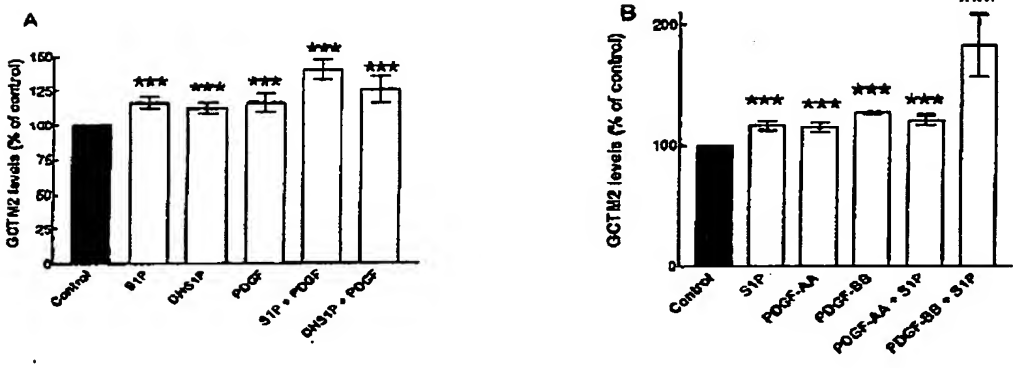
A
GCTM2 levels (% of control)
150
125
100
75
50
25
0
***
Control
S1P
DHS1P
PDGF
S1P + PDGF
DHS1P + PDGF
B
GCTM2 levels (% of control)
200
100
0
***
Control
S1P
PDGF-AA
PDGF-BB
PDGF-AA + S1P
PDGF-BB + S1P

FIG 6

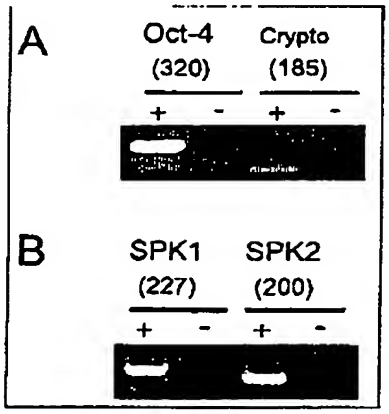

FIG 7

Phillips Ormonde Fitzpatrick

Australian and New Zealand Patent & Trade Mark Attorneys

21 March, 2002

Telephone Contact : Ann Kurts
+612 9929 5400

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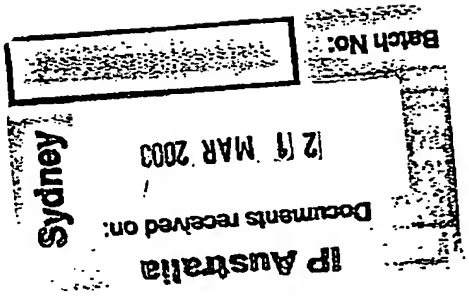

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+613 9614 1944

Facsimile
(03) 9614 1867
(03) 9614 1483

International Facsimile
+613 9614 1867
+613 9614 1483

Email
attorney@pof.com.au

Internet
www.pof.com.au
www.ipmenu.com

Sydney
54 Miller Street
North Sydney 2060
AUSTRALIA

Telephone
(02) 9929 5400

Facsimile
(02) 9929 4511

New Zealand
PO Box 30941
Lower Hutt
New Zealand

Adelaide
45 Grenfell Street
Adelaide 5000
AUSTRALIA

Telephone
(08) 8212 4622

Facsimile